Shiraz University

IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.3 Ser. No.88 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Cytotoxic and apoptotic effects of zoledronic acid on D-17 canine osteosarcoma cell line

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10.22099/ijvr.2024.47475.6877

(Received 7 Jun 2023; revised version 4 Sept 2024; accepted 6 Oct 2024)

Abstract

Background: Pets are exposed to a multitude of carcinogenic substances in the modern world. Consequently, high rates of cancer are observed, particularly in middle-aged cats and dogs. Although bisphosphonates have been incorporated into the treatment of human cancers, there are few animal-specific studies. As the number of cancer cases in animals continues to rise, it becomes increasingly important to evaluate anticancer drugs on a species-specific basis. **Aims:** The present study aimed to examine the impact of zoledronic acid (ZA) on apoptotic pathways in canine osteosarcoma (OSA) cell lines. **Methods:** The apoptotic effects of ZA administration on D-17 canine OSA cells were analysed by determining apoptotic DNA breaks, caspase-3, -8 and -9 levels by ELISA method and Bax/Bcl-2 ratio by qRT-PCR. The effect of ZA on the colony formation capacity of cells was evaluated by crystal violet staining method. The mineral binding capacity of ZA application in cells was investigated by Alizarin Red S staining technique. The change in alkaline phosphatase enzyme activity in OSA cells due to ZA treatment was determined using the colorimetric method. The antimetastatic effect was determined using the wound healing method, which evaluated the migration potential of cells. **Results:** While ZA application did not show a significant cytotoxic effect in the cells in the first 24 h, a decrease observed in the viability of the cells depending on the increasing dose and time. Low dose ZA (1, 5, 7.5, 10 µM) concentrations increased mineral content and alkaline phosphatase enzyme activity. A significant decrease was found in the expression levels of survivin, which determines the cell survival, depending on the dose and time. **Conclusion:** It is expected that our obtained data will contribute to the more effective treatment of the disease by creating different treatment options for clinicians in the light of increasing knowledge in cancer cell biology.

Key words: Apoptosis, Bisphosphonates, Caspase, Survivin

Introduction

Osteosarcoma (OSA), the most common primary bone tumor rooted from the mesenchymal cells, occurs predominantly in the metaphyseal regions of the long bones. OSA accounts for 4% of all malignancies and 85% of skeletal tumors seen in dogs (Al-Khan *et al*., 2020). It is more common in large and giant breeds and seen in dogs aged 2-15 years (Poradowski *et al*., 2021). OSA shows a high degree of malignancy, has a rapid growth rate, and a tendency to settle in the lungs and metastasize in other organs (Szewczyk *et al*., 2015). Despite the advances in the diagnosis and the treatment of OSA, no change has been observed in the prognosis and survival rates of dogs with OSA over the last 35 years (Al-Khan *et al*., 2020). It has been reported that 45% of the dogs with OSA have a 1-year survival rate after diagnosis and treatment. Less than 20% of dogs with OSA can live more than 2 years (Wilson *et al*., 2008). Still more than 20% of patients die due to tumor metastasis and an unresectable tumor (Zhang *et al*., 2015).

A variety of chemotherapy treatment options, including cisplatin, metotreksat, adriamisin, ifosfamid, vincristine, epirubicin, cyclophosphamide and etoposide are typically employed for the treatment of canine OSA (Boerman *et al*., 2012). In veterinary medicine, although it is not an optimal chemotherapy protocol applied, it has been reported that better results are generally obtained in dogs receiving platinum chemotherapy with or without doxorubicin after amputation (Szewczyk *et al*., 2015). In addition, even if healing is provided with these treatments, they cannot prevent the recurrence and the metastatic spread of the tumor, and pulmonary metastasis develops in almost 80% of the treatments (Szewczyk *et al*., 2015; Zhang *et al*., 2015). Therefore, in order to treat OSA, it is necessary to develop new methods which are anti-metastatic, have minimal cytotoxic effects on healthy cells, strengthen bone tissue, inhibit the activity of osteoclasts, and have antiresorvative properties.

In recent years, research has focused on the potential effects of drugs that regulate osteoclast development and

activity on tumor growth through suppression of osteoclasts. Among these drugs, bisphosphonates (BPs) are an important class of molecules to treat small animals and control bone pain and hypercalcemia (Heymann *et al*., 2005; Suva *et al*., 2021).

Zoledronic acid (ZA) is a nitrogen-containing BPs (N-BPs) used in the treatment of various metabolic bone diseases due to its effectiveness in preventing bone loss (Patntirapong *et al*., 2012). Bisphosphonates are first-line treatments for osteoporosis, while ZA is a third-generation nitrogen-containing bisphosphonate. ZA is used to treat osteoporosis, but an increasing number of studies have shown that it also has anti-tumour activities (Liu *et al*., 2021). ZA is widely used in the prevention of bone metastases due to its inhibitory effects on osteoclasts, and has been reported to inhibit primary tumor growth, reduce lung metastases, and increase survival in animal models of OSA (Caraglia *et al*., 2010; Conry *et al*., 2016). Studies have shown that ZA induces apoptosis by increasing the level of reactive oxygen species in some cell lines (Bruzzese *et al*., 2013; Ge *et al*., 2014). It inhibits RANKL production and leads to apoptosis in cancer cells (Cheung *et al*., 2018). It has been reported that ZA increases apoptosis in different human cancer cells (Tassone *et al*., 2003; Koto *et al*., 2010), but studies on veterinary oncology are limited.

Materials and Methods

Ethics statement

No ethical approval was required, as this study required no animal experiments.

Culturing D-17 (CCL-183) canine OSA cell line

Canine OSA cell line D-17 (CCL-183) purchased from the American type culture collection (ATCC, Manassas, VA) was used in our study. D-17 cells were grown in Eagles minimal essential medium (Sigma M4655) supplemented with 10% fetal bovine serum (Sigma F0804), 0.1% Penicillin/Streptomycin 10.000 U/ml (Gibco), 1% non-essential amino acid (Sigma M7145), 0.11 g/L sodium pyruvate. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. D-17 cells were used at passages 2-10.

The effect of ZA on cell viability

D-17 canine OSA cells were seeded at a density of 5 × 10^3 cells per well in 96-well plates and incubated for 24 h to allow the attachment of cells to the plate. The pharmaceutical company (Novartis) provided the ZA preparation, which was used in the practical application. A range of concentrations of ZA (1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, and 100 µM) were applied to D-17 cells and incubated at 37°C for 24, 48, 72, and 96 h. Following a 24-, 48- or 72-hour incubation period, the medium containing ZA applied to the cells was removed, and 100 µL of medium containing a 10% WST-1 (Roche-11644807001) solution was added to each well. The cells were incubated at 37°C for 3 h in 5% CO_2 and 95% humidity and then measured at 450 nm in a microplate reader (Thermo scientific multiscan go microdrop, Finland). Each experiment with four biological replicate was repeated three times. The percentage of cell viability at the indicated doses were calculated to cells without ZA treatment. The IC_{50} value was calculated using the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Determination of viability by crystal violet staining

D-17 cells were seeded at 1.25 × 10^4 cells in 24-well plates and incubated for 24 h. After 48 and 72 h of incubation of D-17 cells treated with ZA, each well was washed with PBS. The cells were fixed with 10% formaldehyde at room temperature for 15 min, then 5% solution of crystal violet prepared in methanol was added to the cells and left for 15 min. After the dye was removed, images of the plates were taken, and the results were evaluated by comparing the colonies form in the wells.

Effect of ZA on mineralization

Alizarin red S staining was performed to determine the effect of ZA on mineralization (Gregory *et al*., 2004). D-17 cells were seeded at 1.25 × 10^4 cells/well in 24-well plates and incubated for 24 h. Cells incubated with various ZA concentration (1 µM, 5 µM, 7.5 µM, 10 µM, 15 µM, 25 µM, 50 µM, 75 µM, and 100 µM) for 48 and 72 h at 37°C in a humidified atmosphere of 5% CO_2. After incubation, cells were fixed with 10% formaldehyde for 15 min at room temperature. Following, cells were washed with PBS and replaced with 0.04 M alizarin red S stain for 20 min on a plate shaker at room temperature. Cells were washed with PBS, and images of the plates were taken.

Effect of ZA on Alkaline Phosphatase (ALP) activity

Cells were plated in medium with ZA (1 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, and 100 µM) at 5 × 10^5 cells/well in 6-well plates for intracellular ALP activity (Nash *et al*., 2015). At 48 and 72 h into the incubation period, cells were harvested using trypsin-EDTA to assess ALP activity. Then, cells were washed with PBS and homogenised using ice-cold RIPA lysis buffer for lysates (Santa Cruz). The resulting lysates were then centrifuged at 1,000 ×g, 4°C for 15 min. The ALP activity levels in the cell lysates were determined by hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate for 5 min in accordance with the Tietz (1983) method. The optical density was measured at 405 nm. The protein amounts in the lysates were determined with the BCA protein kit (Biovision, USA) in accordance with the procedure.

Effects of ZA on cell migration

Wound models were created by scratching more than 5 mm in length each well with 100 µL pipette tips (Rodriguez *et al*., 2005). Then, cell wash PBS removed non-adhesive cells from the plate and applied media

containing ZA (5 µM, 7.5 µM, 10 µM, 15 µM) below IC_{50} doses during incubation time. Immediately after the application, multiple images of the same point were taken and recorded as the 0th h. The wound image was photographed at 0 h, 48 h and 72 h using the inverted microscope (Olympus). The wound gaps were measured through ImageJ software (version 1.49, USA).

Apoptotic effects of ZA on D-17 cells

Quantitative analysis of apoptotic DNA fragments by ELISA method

In order to determine the effect of ZA on apoptosis, analyses was performed at below IC_{50} doses 5 µM, 7.5 µM, 10 µM, and 15 µM concentrations. The analysis was started by lysis of the 10^4 cells incubated with ZA at the determined concentrations for 48 and 72 h. Apoptotic DNA fragments in the nuclear extract from which cell residues were removed by centrifugation were determined by Cell Death Detection ELISA Plus kit, (Roche, Mannheim, Germany) according to the instruction. The sample without ZA (control cells) was accepted as 1.00, and the enrichment factors of the other samples were calculated proportionally.

Determination of apoptotic effects by DNA fragmentation

DNA extraction was performed with the apoptotic DNA Ladder (Roche, Germany) kit after the cells were treated with ZA (5 µM, 7.5 µM, 10 µM, and 15 µM) for 48 and 72 h. DNA fragments were prepared TBE buffer and subjected to electrophoresis with safe DNA dye (GelRed, Bıotıum, USA) on a 1% agarose gel. The agarose gel runs at a low voltage (75 V) for 90 min. Then, the image was acquired using UVP Bioimaging System (Cambridge, UK) imaging system under UV light.

Evaluation of apoptosis by determining caspase-3, caspase-8, and caspase-9 by ELISA

Cells were incubated for 48 and 72 h by applying media containing 5 µM, 7.5 µM, 10 µM, and 15 µM ZA. Cell lysates were prepared with RIPA lysis buffer. Cell lysates were centrifuged at 10.000 ×g for 10 min at 4°C. After centrifugation, caspase-3, -8, -9 levels in supernatants were determined with canine-specific commercial ELISA (FineTest, Canine caspase-3, caspase-8, caspase-9, China) kits suitable for cell cultures.

Determination of gene expressions by RT-PCR

Total RNA isolation and cDNA synthesis

RNA isolation was performed using RNA isolation kit (RiboEX (GeneAll), Korea) from D-17 cells, treated with ZA at 5 µM, 7.5 µM, 10 µM, and 15 µM concentrations for 48, 72 h. All samples were diluted to 1 µg total RNA for cDNA synthesis. Our RNA samples were converted to cDNA using the cDNA synthesis kit (Transcriptor High Fidelity cDNA Synthese kit, Roche) in a Light Cycler Nano Real-Time PCR device by the procedure.

Primers

Primers for Bcl-2, Bax and Bid genes, which are involved in apoptotic pathways, the survivin gene, which is considered to be the gene associated with cell survival, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a reference gene were designed using the Primer 3 program (Table 1). Primers, were obtained from Atlas Biotechnology Company (Turkey) as lyophilized.

qPCR analysis

Quantification for target and housekeeping genes was performed using the Sybr Green PCR master mix kit (Fast Start Essential DNA Green Master kit, Roche) on a LigthCycler®480 thermal cycler (Roche). The same reference gene was used in both conditions. The protocol was performed in triplicate with three biological replicates. Ct values were obtained for each experimental set, resulting from qRT-PCR were averaged. The ΔΔCt method was used to calculate the relative quantitation of gene expression (Livak and Schmittgen, 2001).

Statistical analysis

MS-Excel 2010, GraphPad Prism 5 and SPSS for Windows ver. 22.0 (SPSS Inc., Chicago, IL., USA) package programs were used. In statistical decisions, $P<0.05$ was accepted as an indicator of significant difference. The conformity of the variables to the normal distribution was examined using the Shapiro-Wilk test. Descriptive analyzes were performed as mean and standard deviation for all parameters. The difference between the groups in the parameters was determined by one-way analysis of variance. Tukey test was performed to determine which group caused this difference.

Results

Effect of ZA on cell viability

Cell survival rate percentages were calculated for each concentration relative to the control group using the data obtained from the WST-1 analysis (Fig. 1). A dose effect curve was generated using the GraphPad Prism 5

Table 1: Primer sequences used for qRT-PCR

NCBI reference sequence	Gene	Forward primers (5´→3´)	Reverse primers (5´→3´)	Amplicon length (bp)
NM_001003142.2	GAPDH	AGTCAAGGCTGAGAACGGGAAA	TCCACAACATACTCAGCACCAGC	114 bp
NM_001251938.1	BID	AATTTGCTAGTGTTTGGCTTCCTC	ATCGTCGTAGTCCTCCTTCAG	117 bp
NM_001003011.1	BAX	TTCCGAGTGGCAGCTGAGATGTTT	TGCTGGCAAAGTAGAAGAGGGCAA	79 bp
NM_001003348.1	BIRC5	CCCAGTGTTTCTTCTGCTTCAA	AGAAAGGAAAGCACAACCAGATG	101 bp
NM_001002949.1	BCL2	CATGCCAAGAGGGAAACACCAGAA	GTGCTTTGCATTCTTGGATGAGGG	76 bp

program (Figs. 2A-C). Different doses of ZA on D-17 cells did not show a significant cytotoxic effect during the 24 h incubation period compared with the control (P≥0.05). When the results were evaluated, it was determined that the cytotoxic effect of ZA on D-17 cells increased two fold at 72 h compared to 48 h. The results were statistically significant compared with the control group (P<0.001). The IC_{50} values of ZA treatment on the D-17 cell line were calculated as 82.50 µM, 26.06 µM, and 17.60 µM for the 48th, 72nd and 96th h, respectively.

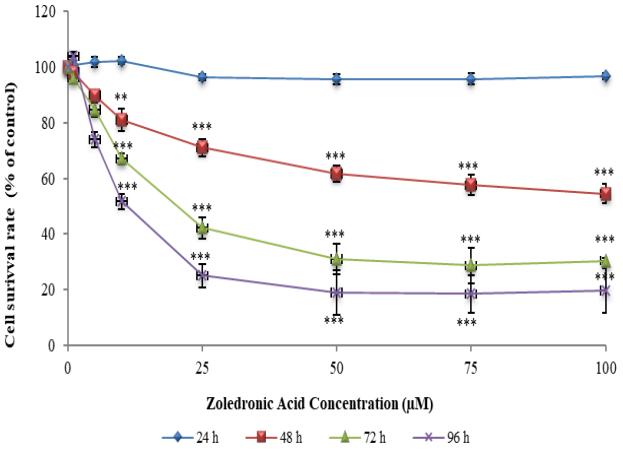


Fig. 1: Percentage of cell survival of D-17 canine osteosarcoma cells at the indicated doses with ZA at the end of the 24, 48, 72, and 96 h incubation periods. ** 0.001≤P<0.01, and *** P<0.001. Percent vitality = [ABS average (each sample)/ABS mean (control)] × 100

Determination of viability by crystal violet staining

ZA applied to D-17 cells dose-dependently increased the cytotoxic effect on the cell, resulting in a reduction of the colony-forming abilities of the cells (Fig. 3). Upon analysis of the effects of ZA, a visible cytotoxic effect was observed in cells treated with 15 µM. When the results of other low doses of ZA applications were compared with the control group, it was found that the cytotoxic effect was relatively low. When high doses of ZA (25 µM, 50 µM, 75 µM, and 100 µM) were compared with the control, cell viability and colony formation were found to be significantly reduced.

Effect of ZA on mineralization

In general, the mineral capacity observed in this study was concentrated and time-dependent. In those groups in which cells were exposed low dose ZA (1 µM, 5 µM, and 7.5 µM), increased mineral capacity was observed compared with that in the control groups during 48 and 72 h of incubation time. However, a higher concentration of ZA (25 µM, 50 µM, 75 µM, and 100 µM) applied to the cells resulted in a decrease in mineral capacity (Figs. 4A-B).

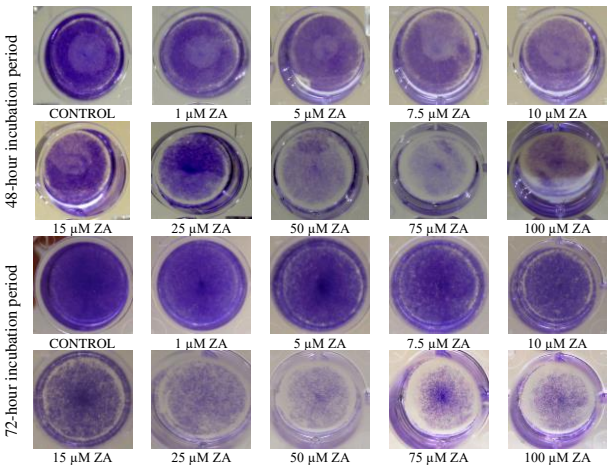


Fig. 3: The effect of zoledronic acid on the viability of cells in the D-17 canine osteosarcoma cell line during the 48 and 72 h incubation

Determination of changes in Alkaline Phosphatase activity of ZA application

The decrease in extracellular ALP activity of D-17 cells, which applied ZA at different doses, was statistically significant after 48 and 72 h of incubation (P<0.001) (Figs. 5A-B). Intracellular ALP activity in D-17 cells treated with ZA at high doses (25, 50, 75, and 100 µM) was observed to significantly decrease in a dose-dependent manner compared with the control group (P<0.001).

Examining the effect on migration of cells

Wound healing results of cells in the D-17 cell line at 48 and 72 h after ZA application are given in Figs. 6A-B. It was determined that 10 and 15 µM ZA inhibited the

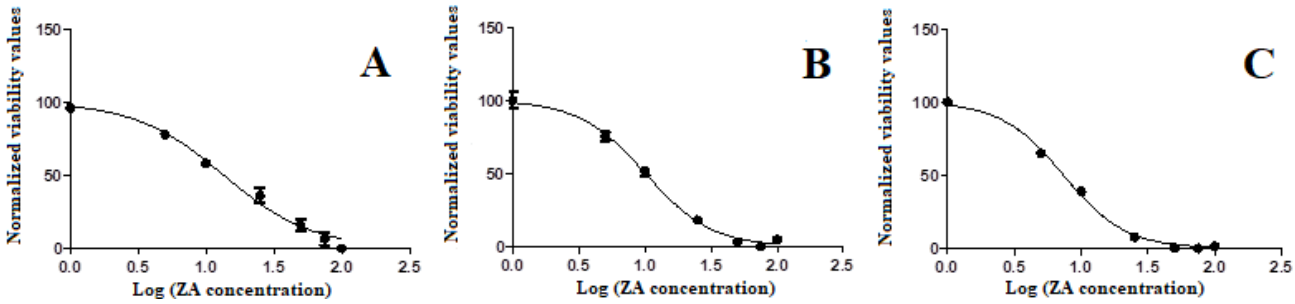


Fig. 2: Graphs of D-17 canine osteosarcoma cell viability due to zoledronic acid doses. **A**: 48 h, **B**: 72 h, and **C**: 96 h

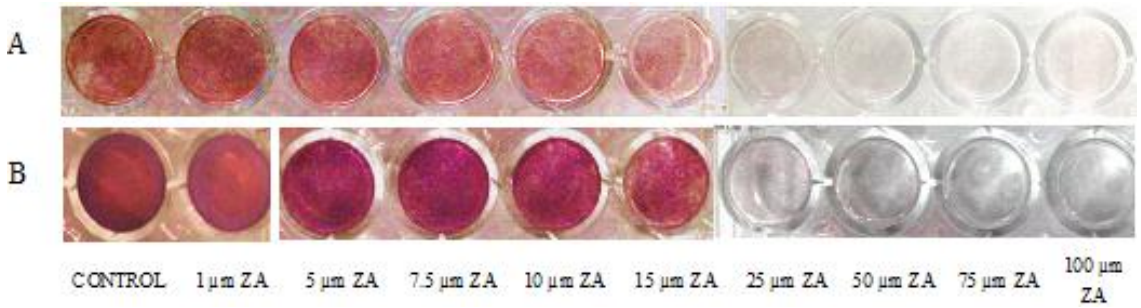


Fig. 4: The effect of zoledronic acid on mineralization of cells during 48 (**A** line) and 72 (**B** line) h incubation in D-17 canine osteosarcoma cell line

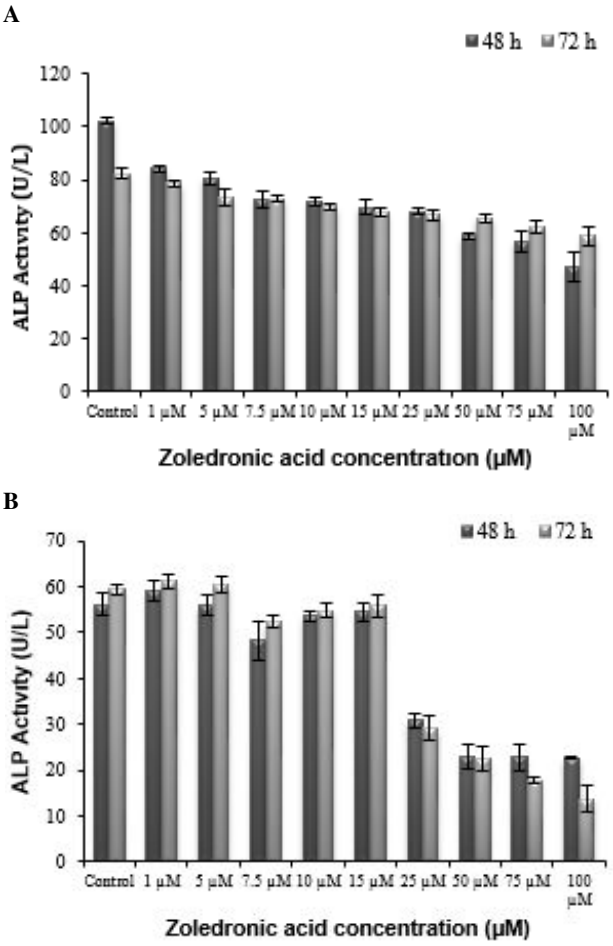
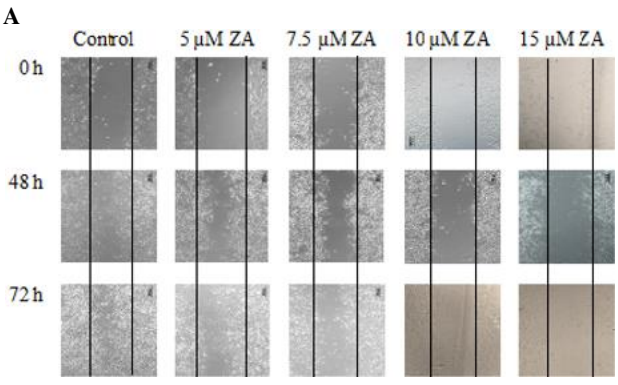


Fig. 5: Changes in extracellular (**A**) and intracellular (**B**) ALP activity depending on the concentration of ZA applied to D-17 canine osteosarcoma cells at 48 and 72 h



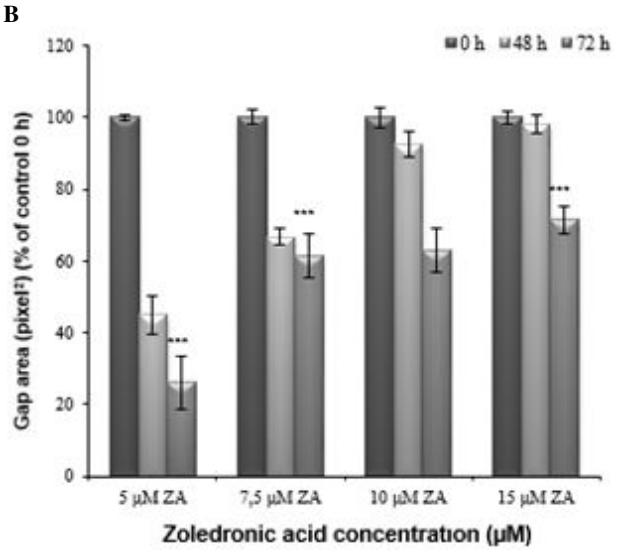


Fig. 6: (**A**) The effect of zoledronic acid on the potential of cells to migrate to the D-17 canine osteosarcoma cell line in a dose- and time-dependent manner (**B**) The ratio of distances between the wounds formed in the cells period compared with 0th h. *** P<0.001

wound's closure by perventing the cell's migration potential. The most effective dose inhibiting the migration of D-17 cells were 10 and 15 µM ZA.

Does Zoledronic Acid induce apoptosis in canine osteosarcoma cells?

Quantitative analysis of apoptotic DNA fragments by ELISA method and display in gel electrophoresis

It was determined by DNA fragmentation analysis with ELISA and gel electrophoresis that cell death occurred through apoptotic pathways in D-17 cells with the application of ZA (Fig. 7A). In applying ZA to D-17 cells, the amount of DNA fragments increased 1.34-fold and 1.57-fold in 5 µM and 7.5 µM applications, respectively, as compared with control cells without ZA treatment (P<0.05). At the same time, in the application of 10 µM and 15 µM ZA on cells, DNA fragmentation increased by 2.13 and 2.32 fold, respectively, as compared with the control group that did not receive ZA (P<0.01). The gel run images of DNA breaks showed no difference between the control group and the 48th h (data not shown). However, DNA breaks were increased in a dose-dependent manner at 72 h after the application (Fig.

7B).

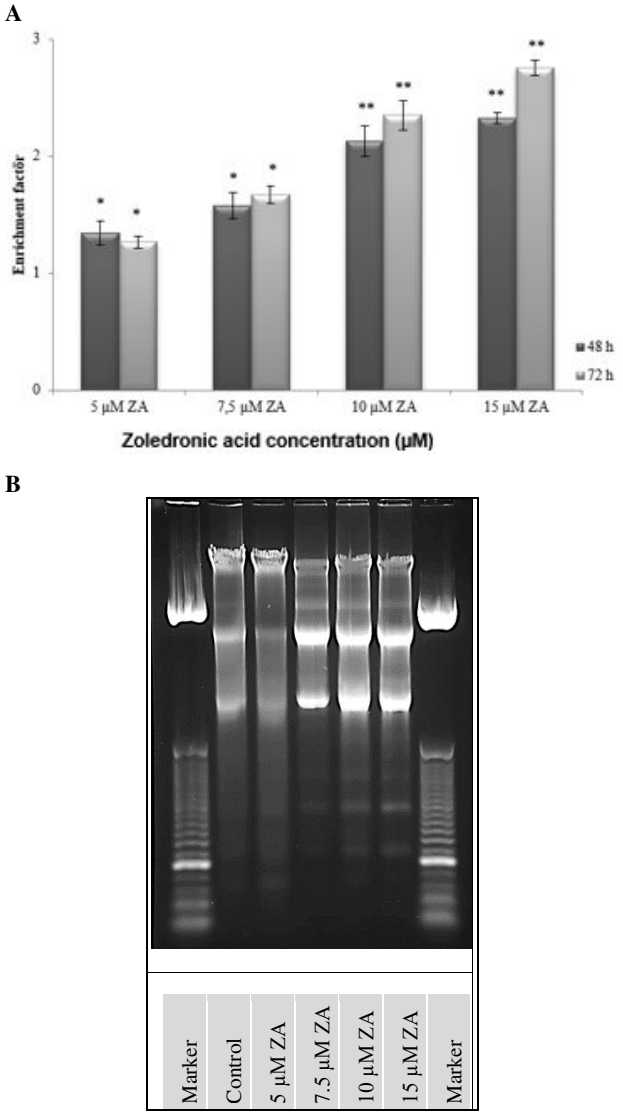


Fig. 7: (**A**) DNA fragmentation analysis of the apoptotic effect of zoledronic acid in D-17 canine osteosarcoma cells (enrichment factor = ABS mean (each sample)/ABS mean (control)). According to the control group, * $0.01 \leq P < 0.05$, ** $0.001 \leq P < 0.01$, and (**B**) Image of DNA fragmentation in 1.5% agarose after 72 h of incubation of D-17 canine osteosarcoma cells with zoledronic acid. Marker: 100 bp DNA molecular weight marker

As a result, apoptosis-induced DNA breaks in the cell with the effect of ZA significantly increased, depending on the dose and incubation time. When evaluated together with WST-1 cell viability data, it was determined that low doses of ZA caused apoptosis without cytotoxic effect. It was determined that the data obtained using the ELISA method, which showed cell death due to apoptosis occurred, were consistent with the images of DNA breaks obtained in agarose gel electrophoresis.

Changes in caspase-3, -8 and -9 levels by application of ZA to D-17 cells

In D-17 cells, after 48 h of incubation, a low increase of 17% was observed in the amount of caspase-3 in 5 µM ZA application, while an increase of 84% and 170% was detected in 7.5 and 10 µM ZA concentrations, respectively. However, the increase in caspase-3 level in 15 µM ZA application is around 40% compared with the control group. 72 h after the application of ZA, the amount of caspase-3 in D-17 cells decreased at 5 and 7.5 µM ZA concentrations compared with the 48th h. Still, there was no statistically significant difference compared with the control group ($P>0.05$). While an increase was observed in both 10 µM and 15 µM ZA application compared with the control group, a decrease was alson determined as compared with the 48th h (Figs. 8A-B).

When the change in caspase-8 levels was examined with the application of different doses of ZA to the D-17 cell line, the difference between all ZA dose groups and the control group was found to be statistically insignificant at the end of both 48 and 72 h of incubation ($P>0.05$) (data not shown).

A low increase of 12.5% and 23% was observed in the caspase-9 amounts of 5 and 7.5 µM ZA concentrations, respectively after 48 h of incubation in D-17 cells compared with the control; however, an increase of 81% was detected in the application of 10 µM ZA. The change in the amount of caspase-9 in D-17 cells 72 h after 5 µM ZA application was found to be statistically insignificant as compared with the control ($P>0.05$). However, the caspase-9 level decreased in 7.5 and 10 µM ZA application compared with the 48th h, while the most significant decrease was observed in 15 µM ZA application (Figs. 8C-D).

The determination of the quantitative expression levels of the genes

When the change in Bax/Bcl-2 ratio in cells treated with ZA was examined, it was observed that at the 24th h, an increase was achieved as 1.36 and 1.69 times for 5 and 7.5 µM concentrations respectively. Also, an increase was achieved as 2.95 and 2.14 times for 10 and 15 µM ZA concentrations respectively, when compared with the control group. Depending on the incubation period, the Bax/Bcl-2 ratio decreased in all doses at the 48th h compared with the 24th h (Fig. 9A).

When the control group and ZA applications were compared, it was determined that there was no significant difference in the expression level of the Bid gene depending on the dose and time. Thus, it was concluded that the Bid gene was not active with ZA administration (Data not shown).

A quantitative change in the survivin gene was observed in the D-17 cell line over the first 24 h. The decrease was 1.34 and 1.59 times for 5 and 7.5 µM concentrations, and 2.49 and 3.02 times for 10 and 15 µM ZA concentrations, respectively, when compared with the control group. The expression of the survivin gene was observed to continue to decrease at the 48th h, depending on the incubation period. In comparison with the control group, the expression of the survivin gene was decreased by 2.57, 4.93, and 5.03 times,

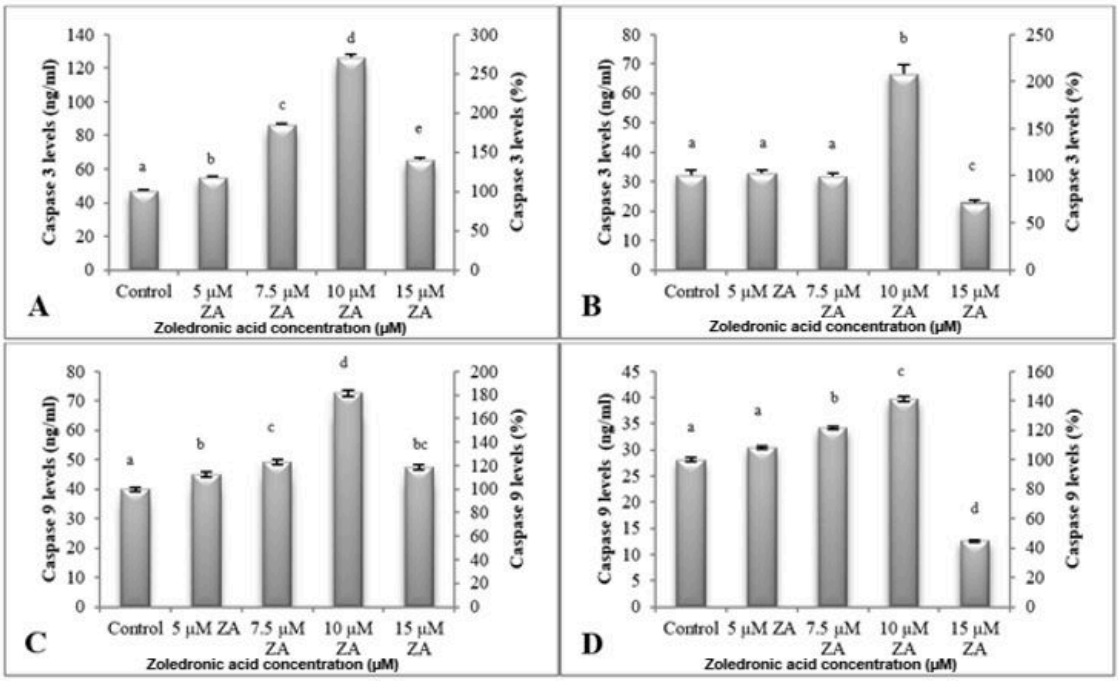


Fig. 8: Graphs of change in caspase-3 and -9 levels depending on zoledronic acid doses (**A**: Caspase-3 level at 48 h, **B**: Caspase-3 level at 72 h, **C**: Caspase-9 level at 48 h, and **D**: Caspase-9 level at 72 h). The difference between means with different letters is statistically significant

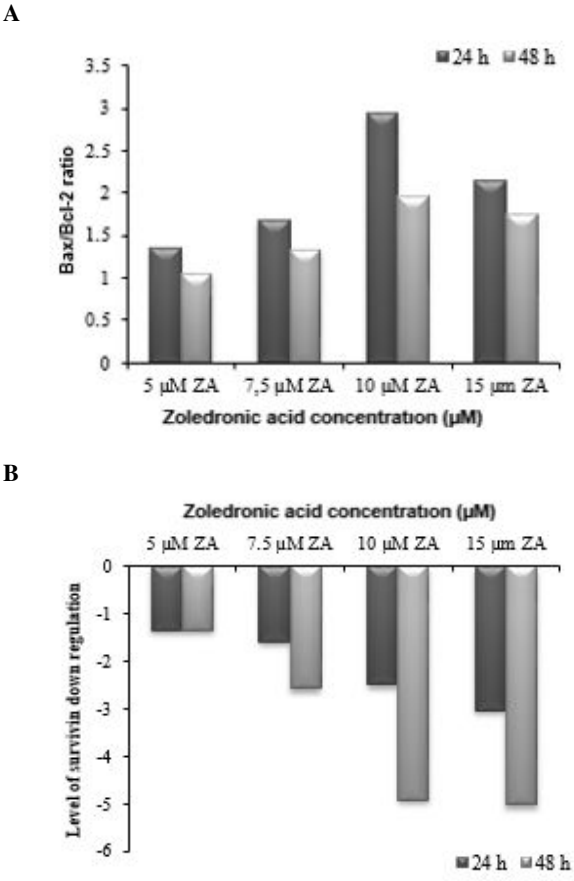


Fig. 9: (**A**) Change in Bax/Bcl-2 ratio depending on dose and duration of zoledronic acid administration according to RT-PCR results, and (**B**) The change in the expression of the survivin gene depending on the dose and duration of zoledronic acid administration according to RT-PCR results

respectively, with the effect of 7.5, 10, and 15 µM ZA at 48 h (Fig. 9B).

Discussion

ZA was preferred in this study because it is one of the most potent N-BPs and is currently being used in the adjuvant treatment of bone metastasis in humans. In addition, there are publications on the effects of ZA alone or in combination with other anticancer agents, especially against human OSA cells (Ryu *et al*., 2010; Christou *et al*., 2023) but studies on veterinary clinical applications of ZA are limited. Smith *et al*. (2023) administered zoledronic acid as a single agent, usually as a single dose *in vivo*, and observed its anticancer effect, but the study was performed in a small number of dogs with OSA.

The investigation of the apoptotic effects of ZA was continued with ZA concentrations (5, 7.5, 10, and 15 µM) below the IC_{50}, where no cytotoxic effect was observed. A number of cell studies, including those conducted on OSA cell lines, have demonstrated that low concentrations of ZA do not exhibit cytotoxic effects on cells (Ryu *et al*., 2010; Patntirapong *et al*., 2012). When the effect of ZA on the colony-forming abilities of the D-17 canine OSA cell line was examined, ZA shows anti-proliferative properties at high doses and prevents cells from coming together, thereby inhibiting the formation of colony forms of cells. The decrease in colony formation causes the communication between cells to gradually decrease, and the factors that induce proliferation are eliminated. It is thought that the effect of ZA on colonization is low due to its low cytotoxic

effect at low doses. These results are in parallel with the results of the WST-1 analysis, which is the cell viability test, and confirm our results.

In our study, after evaluating the data obtained from analyses by alizarin red staining of mineral capacity, it was observed that there was no decrease in mineralization capacity at low ZA concentrations (0-7.5 μM); however, at high concentrations, cell viability was maintained below 50%, while mineralization capacity was strongly inhibited. Similarly, Orriss *et al*. (2009) and Basso *et al*. (2013) evaluated the effect of ZA on the mineralization capacity of human osteoblast MG63 cells and mouse osteoblast cells. They reported that in cells exposed to ZA, mineral nodule formation (MNF) varied depending on concentration and time, and there was a statistically significant decrease in MNF in ZA-treated samples compared with the control groups. Therefore, ZA could affect bone matrix synthesis and mineralization process. The reason why high concentrations of zoledronate inhibit bone matrix mineralization is due to the obvious structural similarity between BPs and mineralization inhibitor pyrophosphates and their direct physiochemical effects on hydroxyapatite crystal dispersal (Orriss *et al*., 2009). Tobias *et al*. (1993) suggested that N-BPs at micromolar concentrations cause apoptosis in osteoblasts *in vitro* conditions and inhibit differentiation in osteoblasts due to their inhibitory effect on mineralization.

MG-63, Saos-2, and U-2 human OSA cell lines (Farley *et al*., 1989; Pautke *et al*., 2004) and mice OSA cell lines (Ali *et al*., 1993) have been shown to produce large amounts of ALP. It is thought that progression, spread or metastasis of OSA will increase osteolysis and ALP. High ALP level has been associated with OSA malignancy and poor treatment methods (Ren *et al*., 2015). In our study, a statistically significant decrease was observed in the level of extracellular ALP in all ZA dose groups compared with the control group at the end of 48 and 72 h of incubation periods. This decrease in intracellular ALP level was determined at concentrations of 25 μM and above at the end of the first 48 h and at concentrations of 7.5 μM and above at the end of the 72 h incubation period. When our results were evaluated, it was seen that ZA inhibited cell differentiation and caused a decrease in ALP level. When this decrease is assessed during the treatment process, it can be argued that ZA may be a suitable agent for the treatment of canine OSA and the prognosis can be followed by serum ALP levels. Studies show that the decrease in ALP activity may also prevent mineralization capacity depending on ZA concentrations (Basso *et al*., 2013). When examined in our results, high dose ZA- and time-dependent decrease in ALP activity shows parallelism with the decrease in mineralization capacity detected with alizarin red.

In our study, the effect of ZA on the migration potential of cells was determined using the scar formation technique, which is a simple and inexpensive method. The concentrations at which the most effective inhibition of migration into the cell, were determined as 10 and 15 μM ZA for 48 and 72 incubation times. Similar to our study, Ory *et al*. (2008) also observed that 10 μM ZA completely blocked the migration of OSRGA (rat OSA) cells after 48 h of wound formation. It can be thought that ZA disrupts the OSA cytoskeleton organization due to increasing doses and inhibits cell migration in this way and has an anti-metastatic effect.

Studies show that ZA induces apoptosis in many cancer types depending on dose and duration *in vitro* and *in vivo* applications (Tassone *et al*., 2003; Ullén *et al*., 2009; Koto *et al*., 2010). However, studies showing that it induces apoptosis in canine OSA, or through which pathways it stimulates are limited (Poirier *et al*., 2003; Fu *et al*., 2011). In order to investigate the presence of DNA fragmentation, its presence in D-17 cells treated with different doses of ZA was investigated both quantitatively by the cell death determining ELISA method and qualitatively by running the genomic DNA obtained from the cells on agarose gel. When our ELISA results were analyzed quantitatively, an increase observed depending on the dose and time, supporting the gel images. Similarly, Evdokiou *et al*. (2003) observed the characteristic genomic DNA fragments in apoptotic cell death when ZA was applied alone to human osteogenic sarcoma cell lines (HOS, BTK-143, G-292) in agarose gel electrophoresis.

Caspase proteins are generally inactive in the cell and when they become active, they drive the cell to death. Since the expression level of the active form of caspase 3 is generally used to reveal apoptosis (Forest *et al*., 2005), information about the level of apoptosis can be obtained depending on the amount of caspase 3. According to our results, ZA appears to cause apoptosis by activating caspase-3. In our study, the increase determined in caspase-3 and -9 levels at 48th h of ZA application to D-17 cells is thought to induce the formation of caspase-9 as initiator caspase and active forms of caspase-9 as effector caspase in the cell. When the results of caspase-8, which is one of the initiator caspases activated due to the stimulation of death receptors, were examined in our study, no statistically significant change was observed in the level of caspase-8 depending on the ZA concentration. It is thought that the administered doses of ZA cause caspase-8-independent apoptosis. The absence of a correspondingly significant change in Bid gene expression levels supports these results.

The balance between proapoptotic and anti-apoptotic proteins may determine the occurrence of apoptosis. Down-regulation of Bcl-2 expression may be reflected by an increase in the Bax/Bcl-2 ratio and an increase in free Bax. Bax then translocates to mitochondria and activates apoptotic cascades (Adams and Cory, 1998). When the results of our study were examined, it was found that the ratio of Bax and Bcl-2 changed in favor of apoptosis in cells treated with ZA. This ratio is an important marker in demonstrating the activation of the mitochondrial apoptosis pathway and is used for this purpose in studies (Yu *et al*., 2013). As a result, it was determined that ZA induced mitochondrial apoptosis by up-regulation of Bax and down-regulation of Bcl-2 in

canine OSA cells the most effective dose for this was 10 μM.

While a more comprehensive study of canine tumors is necessary, Uchide *et al*. (2005) and Kavya *et al*. (2017) demonstrated a significant correlation between survival and tumor malignancy in dogs and overexpression of survivin. Shoeneman *et al*. (2012) reported that inhibition of survivin in Abrams and D17 canine OSA cell lines induced apoptosis, mitotic arrest, and increased caspase activity in the presence of carboplatin and doxorubicin. Similarly, in our study, the quantitative change of survivin gene in the D-17 canine OSA cell line was decreased at all ZA doses administered at 24 h compared to the control, while the decrease was more pronounced at 48 h.

In summary, administered ZA showed antitumor activity by stimulating the caspase-3 and -9 apoptotic pathways of the D-17 canine osteosarcoma cell line. The antitumor effect was confirmed by its effect on Bax and Bcl-2 genes. When the expression of the survivin gene was examined, it inhibited the proliferation of cancer cells by affecting the cell cycle. At the same time, it was found that the migration potential of the cells was reduced and it is thought that it may have an anti-metastatic effect.

An increasing number of studies in veterinary oncology for the treatment of canine osteosarcoma are contributing to the development of various protocols for the management of this serious disease. Cell culture studies are of critical importance in the evaluation of the effects of anticancer agents and combination protocols. In this context, our study sought to investigate the effects of ZA on apoptotic pathways in a canine osteosarcoma cell line. The findings demonstrated that ZA activates apoptotic pathways. ZA was observed to inhibit the proliferation of osteosarcoma cells and affect the cell cycle. In addition, ZA was found to have the potential to reduce the metastatic properties of canine osteosarcoma cells, indicating an anti-tumor spreading effect. All data suggests that ZA could be considered as a potential therapeutic option for the treatment of canine osteosarcoma. Further studies may examine ZA's clinical efficacy and overall safety profile in more detail, expanding its application in veterinary oncology.

Acknowledgements

The manuscript is result of the Doctorate Thesis of the first author. The authors would like to thank the Department of Biochemistry, Faculty of Veterinary Medicine, Adnan Menderes University.

Conflict of interest

The authors declare no conflict of interest.

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